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Recovery of astaxanthin from shrimp cooking wastewater. Optimization of astaxanthin extraction by response surface methodology and kinetic studies.

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Abstract

A protein and astaxanthin- concentrated fraction (R_f) can be recovered from shrimp cooking wastewater by ultrafiltration at 300 kDa, indicating astaxanthin is somehow associated to membrane- retained proteins. Concentrated astaxanthin from shrimp wastewater can be extracted using sunflower oil under milder conditions (T<40ºC) than directly from shrimp exoskeleton. Modeling astaxanthin extraction kinetics at 30ºC revealed the process is consequence of both mass transfer and hydrogen bonding between astaxanthin and oil. The best yields of astaxanthin extraction were obtained using an oil:waste ratio of 3:1 which was not further improved after hydrolysis with alcalase at 45ºC for 30 min (HR_f). The lyophilized concentrate (LR_f) showed two-phase extraction profiles with a much faster pigment recovery observed at 30ºC compared to the liquid form. Astaxanthin from this shrimp by-product has low thermal stability in oil at high temperatures (60 and 70ºC), suggesting the carotenoid is mainly free as a result of the cooking process and not bounded to proteins or lipids as it occurs in its natural form.

Keywords: astaxanthin; sunflower oil; shrimp by-products; extraction kinetics; mathematical modeling; surface response methodology

Introduction

The fish processing industry generates several wastewater effluents (washing, thawing, rinsing and cooking), which involve serious problems of pollution and environmental health. Among these effluents, cooking juice (more than 40% of the total) contains a high saline content and organic load (Cros et al. 2006). Although the effluent composition varies depending on the ratio product/water, the animal species and the cooking duration, they usually show a high chemical oxygen demand (Whala et al. 2009). Consequently cooking wastewaters need to be treated to reduce their pollutant content, thus increasing the cost of the manufacturing process. An alternative to reduce wastewaters processing costs would be the recovery of products with high added value such as proteins, aromas and flavours (Vandajon et al. 2002). Given the carotenoprotein character of the pigmented byproduct from crustacean process wastewaters (Cano-López et al. 1987; Simpson and Haard, 1985), these effluents can also be a possible source of carotenoids.

Astaxanthin (3,3-dihydroxy-ß,ß-carotene-4,4 dione) is a ketocarotene widely used in aquaculture as feed additive for the pigmentation of salmonids meat and shrimp and lobster shells. These animals do not synthesize carotenoids *de novo* and need to ingest these pigments in the diet to lead to their characteristic orange-red coloration. In the marine environment, animals accumulate astaxanthin from the zooplankton, which in turn ingests phytoplankton or microalgae containing the carotenoid synthetized *de novo* (Foss et al. 1987). However, astaxanthin found in the body of aquatic animals can also be a consequence of the conversion through metabolic reactions of other absorbed carotenoids (Matsuno, 2001).

The majority of commercial astaxanthin for aquaculture is industrially produced by chemical synthesis (Rodriguez-Saiz et al. 2010) although its increasing interest, due

to novel applications as nutraceutical in the food, pharmaceutical and cosmetic industries (Del Campo et al. 2007), has led to several studies about its biotechnological production (Domínguez-Bocanegra et al. 2007; Chávez-Cabrera et al. 2010; Nghiem et al. 2009). The microalga *Haematococcus pluvialis* and the yeast *Xantophyllomyces dendrorhous* are the most promising microorganisms regarding the industrial production of astaxanthin, due to their ability to biosynthesize *de novo* high amounts of the pigment (Bhosale and Bernstein 2005). While to date these productions seemed unable to compete with the chemical synthesis, recent publications describe improved processes for large-scale astaxanthin bioproductions (De la Fuente et al. 2010; Li et al. 2011).

Currently, efforts are focused on the search for new natural sources of astaxanthin. In this way, many studies describe the recovery of astaxanthin from shrimp byproducts such as head and body skeleton (Armenta-López et al. 2002; Bi et al. 2010; Sachindra and Mahendrakar 2005). De Holanda and Netto (2006) also reported the obtaining of astaxanthin as a valuable subproduct of the chitin production from shrimp processing waste. In these studies, different methods are used to extract astaxanthin such as vegetable oils (Chen and Meyers 1982; Handayani et al. 2008; Sachindra and Mahendrakar 2005), organic solvents (Sachindra et al. 2006), fermentative process (Sachindra and Bhaskar 2008) and enzymatic hydrolysis (De Holanda and Netto 2006).

During the last years, the application of membrane technology as main method of separation, concentration and purification of valuable compounds from fish processing residual materials has been highly developed (Afonso et al. 2004; Murado et al. 2009; Murado et al. 2010).

In the present study we describe a feasible process using membrane technology for the recovery of astaxanthin from shrimp cooking wastewater. This methodology allows obtaining a protein and astaxanthin- concentrated fraction that can be used as additive in the animal feed industry, while reducing the costs of wastewater treatment. This study also reports the optimized conditions (temperature, time and ratio oil: waste) for carotenoid extraction using sunflower oil and proposes kinetic models that would be helpful for the further scale-up of the process.

Materials and methods

1. Materials

The company Bajamar Séptima, Pescanova Group (A Coruña, Galicia, Spain) kindly provided the cooking wastewater from the industrial manufacturing of shrimp (*Penaeus vannamei*). Shrimp cooking juice was sampled and immediately stored at -18ºC until further use.

2. Analytical determinations

Protein, total nitrogen, total sugar and reducing sugar contents were determined from samples taken before storage. Total nitrogen was determined by the method of Havilah et al. (1977). Soluble proteins were determined using the method of Lowry et al. (1951), total sugar content by the phenol-sulphuric acid method (Dubois et al. 1956), according to Strickland and Parsons (1968) and reducing sugars were quantified by means of a 3,5-dinitrosalicylic reaction Bernfeld (1951). The shrimp cooking wastewater utilized in this work had a pH of 6.07±0.04, a protein content of 1.92±0.08 g/L and a total soluble sugar concentration of 0.21±0.02 g/L.

3. Recovery of astaxanthin by ultrafiltration of shrimp wastewater

The concentration of astaxanthin from the shrimp cooking juice consisted of ultrafiltration-diafiltration using a spiral polyethersulfone membrane (Millipore Prepscale) of 0.56 m^2 with molecular weight cut-off (MWCO) of 300 kDa. The operation mode was the following: an initial phase of ultrafiltration (UF) with total recycling of retentate, immediately followed by diafiltration (DF). During UF, the inlet pressure remained constant to determine the drops of flow rate due to the increased concentration of the retentate and to possible membrane adhesions. The final retentate (after DF) was divided into two batches, one was directly stored at -18°C (R_f) and the other lyophilized (LR_f) and stored at 4ºC for further analysis. Both permeate in the UF and DF phase were discarded after analysis.

The kinetics of UF and DF of the effluent were defined by the protein levels as determined by two procedures, the method of Lowry and the total nitrogen multiplied by 6.25.

4. Enzymatic hydrolysis process

The enzymatic hydrolysis of the concentrated fraction was performed using a commercial protease, alcalase 2.4 L from Novo Co. (Novozyme Nordisk, Bagsvaerd, Denmark) at a ratio of 0.01:1 (U/mL) enzyme/substrate. The pH of the retentate was adjusted to pH 9.0 using 5 mM Britton-Robinson buffer and proteolysis was carried out in a water bath with soft agitation at 45ºC for 30 min. The hydrolysate (HR_f) was stored at -18ºC until further use.

5. Combined effect of temperature, heating time and oil:waste ratio on the astaxanthin extraction

A second-order rotatable design, based on three variables at five levels (Akhnazarova and Kafarov 1982; Box et al. 2005), was used to study the combined effect of temperature (*T*), time (*t*) and ratio oil: waste (*R*) on the yield of recovered astaxanthin from shrimp process wastewater. The joint effect of the three variables was studied in the R_f fraction.

The experimental domains of each variable were 40-100ºC for *T*, 30-300 min for *t* and 1.0-3.0 for *R*. The design consisted in 20 experiments with four (2^2) factorial points, four axial points to form a central composite design with α = 1.682 and 6 center points for replication. The experimental domain and codification of the variables are shown in Table 1. Experimental data were fitted to the following empirical model with the yield of astaxanthin as dependent variable:

$$Y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} x_i^{\,2} + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ij} x_i x_j \qquad [1]$$

Statistical significance of the coefficients was evaluated by the Student's t-test (α = 0.05). Consistency of the model was tested by the Fisher's F-test (α = 0.05), using the following mean squares ratios:

	the model is acceptable if
F_1 = Model / Total error	$F_1 \geq F_{den}^{num}$
F_2 = (Model + Lack of fitting) / Model	$F_2 \leq F_{den}^{num}$
F_3 = Total error / Experimental error	$F_3 \leq F_{den}^{num}$
F_4 = Lack of fitting / Experimental error	$F_4 \leq F_{den}^{num}$

Data fitting, parametric estimation performed by minimization of the sum of quadratic differences between experimental and model-predicted values, and significance tests

both for parameters and model, were performed with the *Microsoft Excel* spreadsheet.

6. Extraction of astaxanthin using sunflower oil

The extraction of astaxanthin in sunflower oil was carried out from the final retentate (R_f) but also two other pre-treated samples were studied as astaxanthin sources. For this purpose, R_f was hydrolysed using alcalase (HR_f) and also lyophilized (LR_f) in order to test if the carotenoid was more available to sunflower oil in any of these forms.

Extraction from both R_f and HR_f was performed using the optimized conditions defined by a second-order rotatable design, as previously described. In case of LR_f, the ratio oil:waste was increased to 100:1, an adequate relation due to the increased concentration of the carotenoid as a consequence of the freeze-drying process. In the latter fraction, the extraction was studied at different temperatures: 30, 40, 50 and 60ºC. Extractions were carried out in stirred 250 mL flasks and appropriate R_f or HR_f volumes or LR_f masses were added to sunflower oil preheated at the appropriated temperature. Duplicate samples were removed after different incubation times. Then samples were filtered through washed glass wool, centrifuged at 5000 *g* for 15 min and the pigmented oil layer from the supernatant was recovered. The astaxanthin concentration was measured spectrophotometrically at the λ_{max} (487 nm: A_{487}) and the carotenoid yield as astaxanthin, for liquid (μg/mL) or solid (μg/g) samples, was determined using the following equation (Sachindra and Mahendrakar 2005):

$$Y = \frac{A_{487} \times V_{oil} \times 10^6}{100 \times V_w \times E} \qquad [2]$$

where,

Y is the astaxanthin yield per volume of bulk liquid (µg/mL) or per shrimp waste mass (µg/g); V_{oil} is the volume of recovered pigmented oil; V_w the volume of waste (for R_f and HR_f samples) or the weight of lyophilized powder (for LR_f samples) and E the specific extinction coefficient.

Finally, the effect of the addition of butylated hydroxyanisole (BHA) or ethoxyquin (ETQ) at 200 mg/L on the astaxanthin extraction was also studied in both R_f and LR_f.

7. Mathematical modeling of extraction kinetics

Recently, Handayani et al. (2008) have proposed a mass transfer kinetic model that described the dynamics of astaxanthin extraction using vegetable oil. The proposed equation is a mechanistic model based on the idea that mass transfer mainly controls the extraction of astaxanthin in oil:

$$Y = Y_e\left[1-\exp\left(-k_L a t\right)\right] \qquad [3]$$

where,

Y and Y_e are the astaxanthin yield in bulk liquid and at equilibrium per volume (µg/mL) or per mass of shrimp waste (µg/g), respectively; t is the time of extraction process (min); $k_L a$ is a volumetric mass transfer coefficient (min^{-1}).

These authors also applied a psedudo-second-order model that successfully described their experimental data (Handayani et al. 2008). A Langregan-type equation would account for the esterification between hydroxyl groups in free astaxanthin and fatty acids in sunflower oil that might take place during the extraction

process. Considering that the concentration of astaxanthin at the beginning of the extraction process is zero and rewriting the equation in terms of yield:

$$Y = \frac{Y_e^2 k_A t}{\left(1 + Y_e k_A t\right)} \qquad [4]$$

where,

Y and Y_e are the astaxanthin yield in bulk liquid and at equilibrium per volume (µg/mL) or per mass of shrimp waste (µg/g), respectively; t is the time of extraction process (min); k_A is a reaction constant (min^{-1}).

However the kinetic profiles of astaxanthin extraction reported by Handayani et al. (2008) and those presented in this paper for the 300 kDa lyophilized retentate fraction (LR_f) describe biphasic behaviour. In both cases, the time-course of astaxanthin yield in vegetable oil shows an initial period of rapid pigment transference followed by a slower extraction phase. This, in terms of mathematical modeling, can be easily described using the sum of two mass transfer kinetic models (biphasic model), with different volumetric mass transfer coefficients and yields at equilibrium (Y_{e1} and Y_{e2}):

$$Y = Y_{e1}\left[1 - \exp\left(-k_{L1}at\right)\right] + Y_{e2}\left[1 - \exp\left(-k_{L2}at\right)\right] \qquad [5]$$

where,

Y_{e1} *and* Y_{e2} are the astaxanthin yields per mass of shrimp waste (µg/g), of the first and second phase, respectively; t is the time of the extraction process (min); $k_{L1}a$ and $k_{L2}a$ are the volumetric mass transfer coefficients of the first and second phase, respectively (min^{-1}).

Considering that the sum of both Y_{e1} and Y_{e2} is the maximum yield of extraction achieved (Y_m), and rewriting Eq. 5 in terms of a global process with a single yield at equilibrium (Y_e), we have:

$$Y = Y_e\left[1-\exp\left(-k_{L1}at\right)\right]+\left(Y_m-Y_e\right)\left[\left(1-\exp\left(-k_{L2}at\right)\right)\right] \qquad [6]$$

For comparative purposes, data were normalized by assigning a value of 1 to the higher yield of astaxanthin extracted from each fraction (R_f, HR_f and LR_f) under the experimental conditions assayed in each case.

8. Numerical and statistical methods

Fitting procedures and parametric estimates from the experimental results were performed by minimisation of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro '*Solver*' of *Microsoft Excel XP* spread sheet. Then, confidence intervals from the parametric estimates (Student's *t* test) and consistence of mathematical models (Fisher's *F* test), both with a α=0.05, were determined using '*SolverAid*' macro, which is freely available from de Levie's Excellaneous website: http://www.bowdoin.edu/~rdelevie/excellaneous/.

Results and discussion

1: Ultrafiltration of shrimp wastewater

The ultrafiltration-diafiltration process with a molecular cut-off at 300 kDa showed a high retention of astaxanthin despite the low molecular weight (597 Da) of this pigment. In fact, during the ultrafiltration phase, the initial permeates showed slight

yellowish coloration, completely disappearing after diafiltration and leading to an intense colored retentate.

These results suggest that aggregation phenomena are occurring due to the hydrophobic properties of astaxanthin. It is known that astaxanthin from the shell matrix of crustaceans is mainly found esterified or complexed with proteins (Matsuno 2001). Therefore, astaxanthin in the retentate must be forming polymeric aggregates (Velu et al. 2003) and/or bound to macromolecules, mainly proteins, that are retained during ultrafiltration using the reported cut-off membrane.

In the diafiltration with constant volume (filtration flow = water intake flow), the concentration (or the total amount) of a permeable solute in the retentate follows a first order kinetics (Amado et al. 2013):

$$C = C_f + C_0 \exp\left[-(1-s)D_r\right] \qquad [7]$$

where,

C is the concentration of the permeable solute in the retentate, with C_0 as initial value. C_f is the final asymptotic value if only a part of a polydisperse solute is permeable. Thus, when we use normalized values (%): $C_0 + C_f = 100$, with $C_f = 0$ if all solute is permeable. s, specific retention of the solute. It varies between 0 (the solute is filtered as the solvent) and 1 (the solute is totally retained). D_r, relative diavolume: volume of added water/constant retentate volume.

This equation satisfactorily described the kinetics of protein diafiltration process with a molecular cut-off at 300 kDa (Fig. 1). The values of the coefficients were C_f = 75.9% and s = 0.381, what means a rather high retention of the protein, and also a specific retention that would demand a relative diavolume of 5.7 to eliminate a 99% of permeable protein. In a common diafiltration, with an initial volume of 2 L of

concentrated shrimp wastewater and working with a relative diavolume of 5, at 50–55 ºC and 2 atm (~30 psi), the protein concentration in the retentate can be maintained around 15-20 g/L, with a filtrate flow that decays a 40–45% during the process and maintains an average value of 325 ml min^{-1} m^{-2} (data not shown). Under these experimental conditions the values of protein calculated by Lowry or total nitrogen x 6.25 were almost indistinguishable (Figure 1).

These results indicate a high retention of peptidic material after ultrafiltration of shrimp cooking wastewater despite the heat treatment during shrimp processing. Accordingly, the 300 kDa concentrated fraction could be used as a supplement for animal diets due to its high astaxanthin and protein content. In the same way, Pérez-Santín et al. (2013) obtained a concentrate rich in lipids and proteins with crustacean aroma, attractive orange colouring and antioxidant and ACE-inhibitory capacities making it attractive for the formulation of feeds or functional foods.

2: Enzymatic hydrolysis

The use of proteolytic enzymes has been widely reported to disrupt the protein-carotenoid complex and increase astaxanthin extraction from solid shrimp by-products (De Holanda and Netto 2006; Sowmya et al. 2011). With this purpose, in a preliminary experiment the hydrolysis conditions using alcalase were optimized to maximize the astaxanthin recovery without compromising its stability. Different temperatures (35, 45 and 55ºC) and times (30, 60, 90 and 120 min) of hydrolysis were assayed, maintaining a constant ratio of 0.01:1 (U/mL) enzyme: substrate. After each incubation time, samples were withdrawn and quickly cooled down in an ice-water bath for 5 min to inactivate the protease. Then astaxanthin was then extracted in sunflower oil at 70ºC for 30 min, as previously described. The extraction

temperature was selected according to the optimal conditions reported in the literature for the extraction of carotenoids from shrimp waste with vegetable oils (Sachindra and Mahendrakar, 2005).

Results are shown in Figure 2 where the yields of astaxanthin recovery were calculated according to equation [2]. The highest recovery of astaxanthin under these conditions (70ºC, 30 min) is obtained at 45ºC, falling a 70% on average when the temperature of hydrolysis is 35ºC or 55ºC. Moreover, the astaxanthin yield decreases correlatively with the incubation at all temperatures tested. At the optimum temperature, the recovery of astaxanthin decreases about 36% when the reaction time increases from 30 to 120 min. Taking into account these results, the hydrolysis conditions selected were the following: 30 min at 45ºC using a ratio of 0.01:1 (U/mL) alcalase: substrate.

3: Combined effect of temperature, heating time and oil: waste ratio on the extraction of astaxanthin

The effect of temperature, heating time and ratio oil:waste, on the yield of astaxanthin recovery are important factors that must be considered for a further scale-up of the process. Although the combined effect of these variables can be studied using a one-factor-at-a-time approach, this methodology cannot predict the optimal reaction conditions, ignores interactions and may lead to misleading conclusions. In this regard, experimental design methodologies (Box et al. 2005) are more efficient than one-factor-at-a-time. Response surface methodology uses statistical and mathematical techniques to evaluate the combined effect of factors instead of single factors at different times.

Factorial design methodologies have been successfully applied in the extraction of astaxanthin using vegetable oils (Sachindra and Mahendrakar 2005) and organic solvents (Sachindra et al. 2006). In this work, a second-order rotatable design, based on three variables at five levels (Akhnazarova and Kafarov 1982; Box et al. 2005) was used to study the combined effect of temperature (*T*), time (*t*) and ratio oil:waste (*R*) on the yield of astaxanthin recovery. The experimental domain is shown in Table 1, being temperatures and ratios oil:waste selected according to previous reported conditions for the extraction of astaxanthin using vegetable oils (Sachindra and Mahendrakar 2005). Applying the significance criteria specified in the materials and methods section, the empirical model obtained for the theoretical yield of extracted astaxanthin (*Y*) as a function of the three processing variables was:

$$Y = 8.23 - 1.53T + 0.69R - 1.15tT + 1.11tR + 0.53tTR - 0.43T^2 \qquad [8]$$

The response surfaces obtained varying two independent variables, when the third variable is kept at a constant value, are depicted in Figure 3 and the complete statistical analysis is shown in Table 2. The analysis of variance indicates that the model is significant (α= 0.05) and the adjusted R^2 value shows a good correlation with the experimental data. Besides, according to the statistical analysis, all the parameters in Eq. [8] were significant.

The response surface for carotenoid yield as a function of temperature and ratio oil: waste (Figure 3, left) indicates that the extraction yield increases linearly with the oil: waste ratio. At high temperatures, the response increases notably (96% within the experimental domain) with the proportion of extracting agent. By contrast, at low extraction temperatures, the differences obtained on the carotenoid yield by varying

the phase relationship are much lower (20%). It should also be noted that at high temperatures and low phase relationships, a degradation of the pigment is observed resulting in practically null values of recovered astaxanthin. Figure 3 (right) further confirms these results since it shows the increase in the extraction time has an effect on astaxanthin recovery only at low temperatures. This result also agrees with those reported by Pu et al. (2010), who found shrimp astaxanthin degradation in flaxseed oil was significantly influenced by temperature, with increased degradation rates at 50 and 60 °C compared to 30 and 40ºC using a 1:1 phase relationship.

Although an absolute maximum response was not achieved within the experimental domain, maximal yields can be obtained at low temperature (< 40ºC), high oil: waste ratio (3:1) and high incubation time (> 4 h). Our results also suggest the extraction could be performed at lower temperatures (25-30°C) without appreciable loss in astaxanthin yield and even improving pigment recovery.

Interestingly, our results reveal astaxanthin can be recovered from shrimp cooking wastewaters using milder conditions than the usual high temperatures (Sachindra and Mahendrakar 2005) and organic solvents (Sachindra et al. 2006) utilized for the extraction of astaxanthin from crustacean shells. The fact that astaxanthin is more easily extracted from the liquid effluent than from solid by-products is likely to be due to the cooking process. In fact, several authors suggest that cooking can break the carotenoid–protein complex, releasing the carotenoid compounds and facilitating its extraction (Hornero-Méndez & Mínguez 2007; Mezzomo et al. 2011).

4: Mathematical modeling of astaxanthin extraction kinetics

Optimal values from the factorial design were applied to further improve astaxanthin yield and so extraction kinetics were performed at low temperature (30ºC) and

increasing extraction times. Extraction kinetics from R_f and HR_f at different oil:waste ratios and their predicted profiles using equations [3] and [4] are shown in Figure 4. All parameters were statistically significant (t-Student test, α=0.05) and the predictive ability of both equations was high with a goodness of fit of not less than 0.970 (Table 3).

Nevertheless, the psedudo-second-order model (equation [4]) showed better correlations (R^2) than the mass transfer kinetic model (equation [3]) at all oil:waste ratios. Handayani et al. (2008) also observed better adjustment of equation [4] to the extraction kinetics of shrimp waste in palm oil, which they attributed to the reaction between the hydroxyl groups in astaxanthin with fatty acid. According to these authors, the extraction process is a consequence of both mass transfer and hydrogen-bonding between astaxanthin and oil.

Higher astaxanthin yield at equilibrium (Y_e) was found for R_f fraction with the increase of oil:waste ratio, whereas identical Y_e values were obtained for HR_f at the three assayed oil:waste ratios (Table 3). According to these results, the lowest oil:waste ratio might insufficient to allocate globular proteins in R_f which would tend to be more retained in the oil-water interphase. Astaxanthin can then be partially partitioned between the oil and the interphase. Owing to the excluded-volume interactions (Mazzola et al. 2008) between the carotenoid and these proteins, lower astaxanthin concentrations can be recovered in the oily phase. On the contrary, peptides in HR_f are easier to order and tend to go into the aqueous phase and so astaxanthin could be more easily separated after filtration and centrifugation.

On the other hand, kinetic constants from equations [3] and [4] (k_La and k_A) were greater when a 2:1 ratio was used for oil extraction (Table 3), suggesting this phase

relationship is optimal for mass transfer and reaction between astaxanthin and fatty acids in both R_f and HR_f.

The effect of increasing temperatures on the pigment extraction kinetics from LR_f was also studied. Experimental trends showed the existence of two phases along extraction time, that is, two mass transfer phenomena with different rates (Figure 4). Such behaviour could be due to astaxanthin existing in different forms dependent on the affinity, degree or strength of pigment- protein interactions and also to the presence of free astaxanthin (Pérez-Santín et al. 2013). These profiles made it necessary to use a biphasic equation as [5] to more adequate adjust the experimental data than equation [4]. And in fact, as can be seen in Table 5, the determination coefficients were higher for equation [5] than [4]. The maximum yield of extraction (Y_m) was dependent with temperature, and so lower Y_m values were obtained with temperature increase. This result is in concordance with those using the response surface approach where maximal yields were achieved at low temperatures (< 40ºC). According to the literature, astaxanthin in its free form is unstable and extremely sensitive to factors such as light, oxygen, acidity, and heat (Mezzomo et al. 2011), so these results also support the hypothesis that cooking can break the carotenoid–protein complex and so astaxanthin from cooking wastewater could be mainly in its free form. Moreover, extraction at 30 ºC was much faster from LR_f than from either R_f or HR_f, as can be seen in view of kinetic constants from equation [4] (Tables 3 and 4).

Finally, extraction kinetics in the presence of synthetic antioxidants were performed in order to study the effect these compounds had on astaxanthin recovery. A concentration of 200 mg/L was selected according to commonly used doses in seafood feeds (range of application: 10-150 mg/kg). According to our results (Figure

6 Table 5), addition of either BHA or ETQ improved asthaxantin extraction in sunflower oil. Although different behaviours were observed depending on whether astaxanthin was extracted from R_f (Figure 6A) or LR_f (Figure 6B). The addition of BHA and ETQ significantly ($P<0.05$) increased astaxanthin extraction compared to the control when the pigment was extracted from the liquid sample (R_f), although these differences were not significant ($P>0.05$) in LR_f. However a slower extraction is also observed when performed in the presence of either of two antioxidants (Table 5), suggesting they have a stabilizing effect on astaxanthin that in turns explains the improved extraction observed in the water-oil system. These results show antioxidant addition does not only improve carotene stability during storage (Sachindra and Mahendrakar 2005), but it can also increase the yields of oil extraction.

Conclusions

Our results demonstrate astaxanthin can easily be recovered from shrimp processing wastewaters by UF at 300 kDa. Optimal pigment recovery were obtained at low temperature (< 40ºC), high oil: waste ratio (3:1) and high incubation times (>4 h).

Further analysis of extraction kinetics performed at 30ºC showed astaxanthin recovery is a consequence of both mass transfer and hydrogen bonding between astaxanthin and oil. No improvement in carotenoid yield was observed after hydrolysis with alcalase at 45ºC for 30 min. The lyophilized concentrate wastewater showed a two-phase extraction and at 30ºC was much faster than from the liquid form. Astaxanthin from this shrimp by-product showed low thermal stability in oil at high temperatures (60 and 70ºC), suggesting the carotenoid is mainly free as a result of the cooking process and not bounded to proteins or lipids as it occurs in its natural form. Nevertheless, the retention of the pigment at 300 kDa indicates that

astaxanthin is somehow associated to proteins retained in the ultrafiltration membrane.

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Table captions

Table 1. Experimental domain and codification of independent variables in the factorial design.

Table 2. Results of the experimental plan of the extraction of astaxanthin with sunflower oil according to equation [1] and analysis of significance of the proposed model.

Table 3. Parametric estimations and determination coefficients of equations [3] and [4] applied to the extraction of astaxanthin from the retentate obtained by UF-DF of shrimp cooking wastewaters, before (R_f) and after hydrolysis with Alcalase 2.4 L (HR_f). Extractions were carried out at 30ºC using different ratios oil:waste (1:1, 2:1 and 3:1).

Table 4. Parametric estimations and determination coefficients of equations [4] and [5] applied to the extraction of astaxanthin from the lyophilized retentate (LR_f) obtained by UF-DF of shrimp cooking wastewaters. Extractions were performed at different temperatures using a 100:1 ratio oil:waste.

Table 5. Parametric estimations and determination coefficients of equations [4] and [5] applied to the extraction of astaxanthin from the retentate obtained by UF-DF of shrimp cooking wastewaters before (R_f) and after lyophilization (LR_f), respectively. Extractions were performed at 30ºC in presence of two antioxidants (BHA and

ethoxyquin, ETQ) and without (control), being other extraction conditions described in the text.

Figure captions

Figure 1. Ultrafiltration-diafiltration kinetics of shrimp (*Penaeus vannamei*) cooking wastewater using a polyethersulfone membrane with MWCO at 300 kDa. Left: concentration of retained protein in linear relation with the factor of volumetric concentration (fc) showing experimental data (points) and theoretical profiles (discontinuous line). Right: progress of protein (O) and nitrogen (●) retention with the increase of diavolume from DF process (D). For clarity, confidence intervals (in all cases less than 5% of the experimental mean value; α = 0.05; n = 2) were omitted.

Figure 2. Recovery of astaxanthin in sunflower oil from hydrolysates of the 300 kDa concentrated fraction (R_f). Hydrolysis were perfomed at different temperatures: 35 (△), 45 (O) and 55ºC (●).

Figure 3. Response surfaces of the combined effect of temperature (*T*) and ratio oil:waste (*R*), left, and temperature (*T*) and time of extraction (*t*), right, on the predicted yield of extracted astaxanthin (*Y*) according to Eq. [8].

Figure 4. Astaxanthin extraction kinetics (30ºC) from R_f (●) and HR_f (▲) fractions, using increasing oil:waste ratios: 1:1 (A); 2:1 (B) and 3:1 (C). Experimental data (points) and fittings to equations [3] (– – –) and [4] (–) are shown.

Figure 5. Kinetics of astaxanthin extraction from the 300 kDa lyophilized retentate (LR_f) from shrimp cooking wastewater at different temperatures: 30 (●), 40 (■), 50

(◆) and 60ºC (▲). Experimental data (points) and fittings to equations (– – –) and [5] (–).

Figure 6. Kinetics of astaxanthin extraction from the R_f (A) and LR_f (B) fractions, without (●) and with 200 mg/L of BHA (■) or ethoxyquin (▲). Experimental data (points) and fittings (lines) to equations [4] (A) and [5] (B).

Figure
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Figures

Figure 1

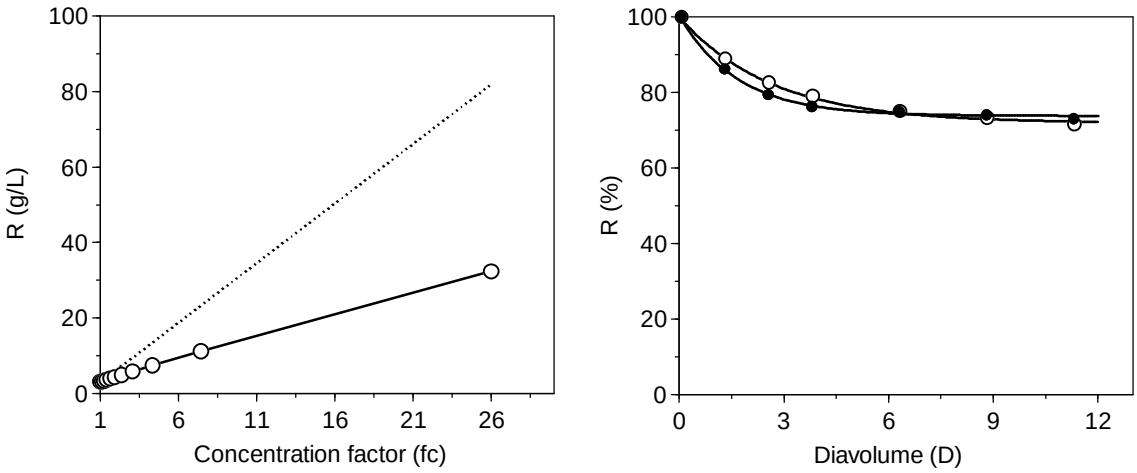


Figure 2

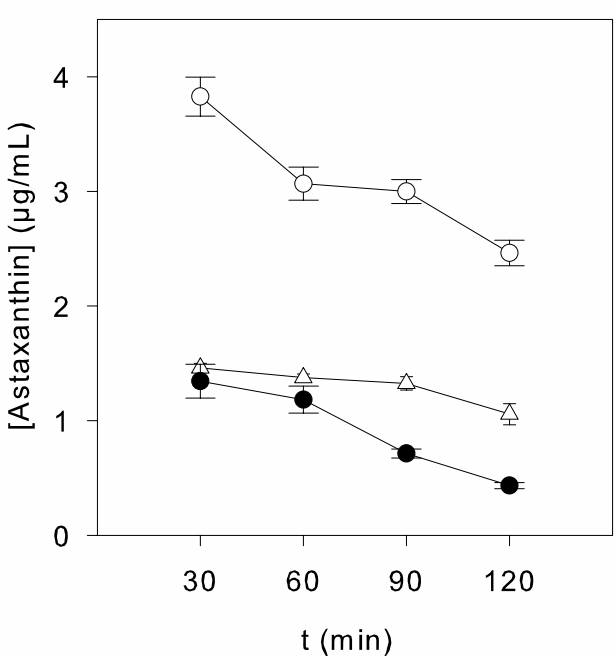
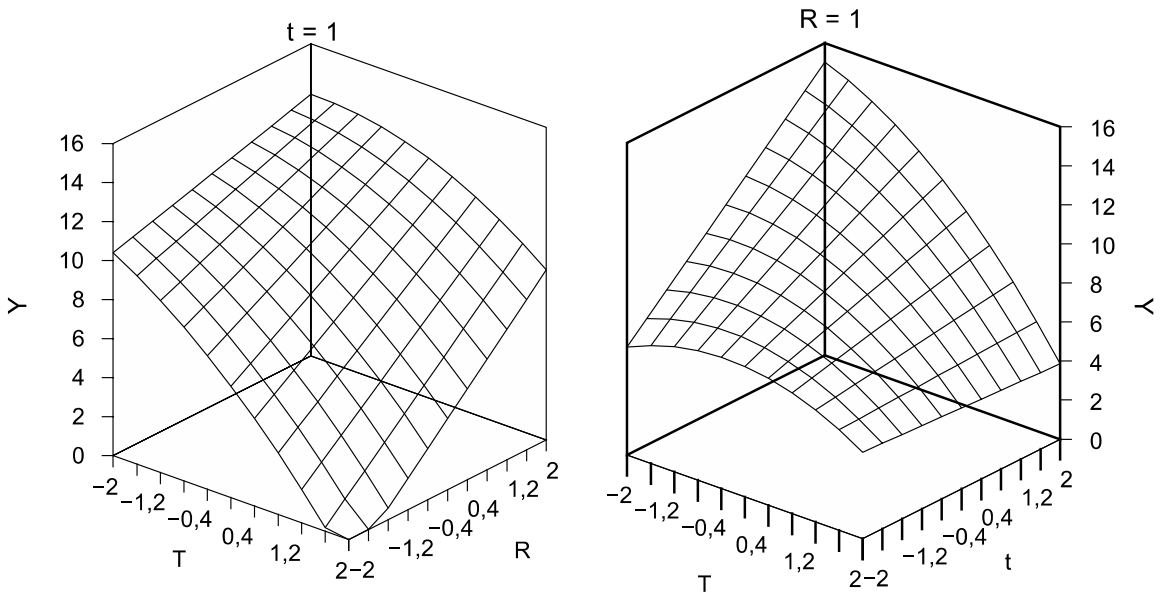


Figure 3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T
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−1,2
−0,4
0,4
1,2
2
R
R = 1
Y
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14
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10
8
6
4
2
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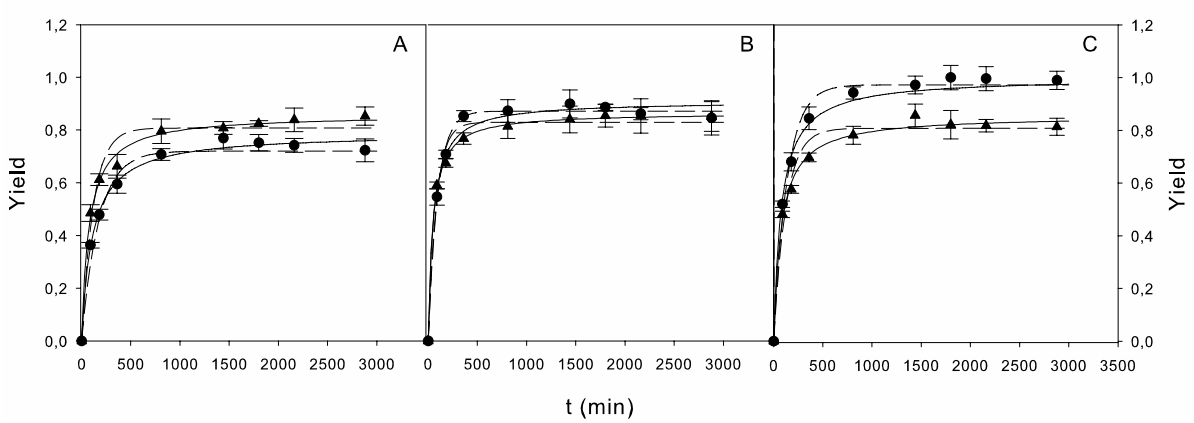
Figure 4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500
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1500
2000
2500
3000
3500

Figure 5

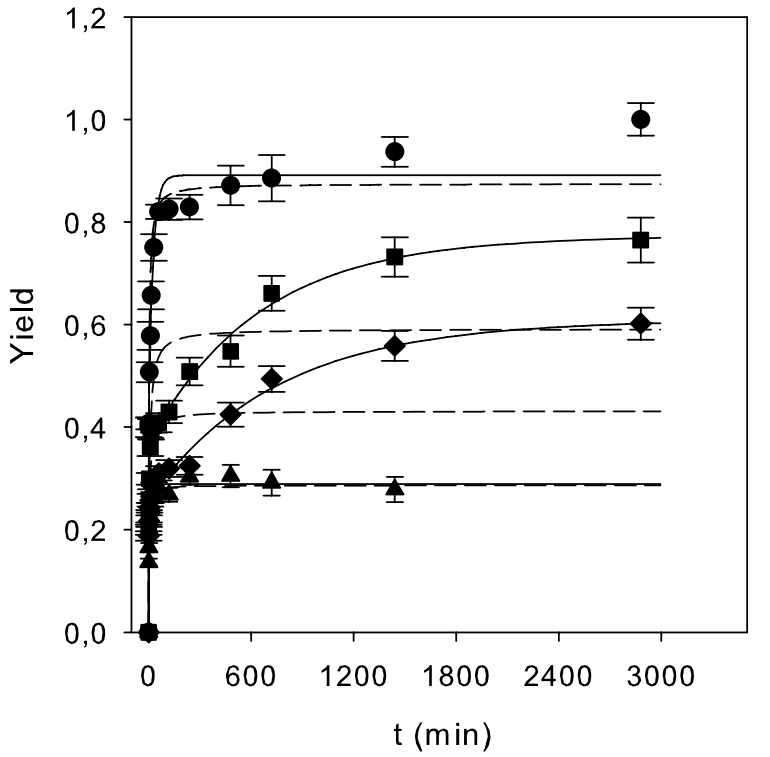
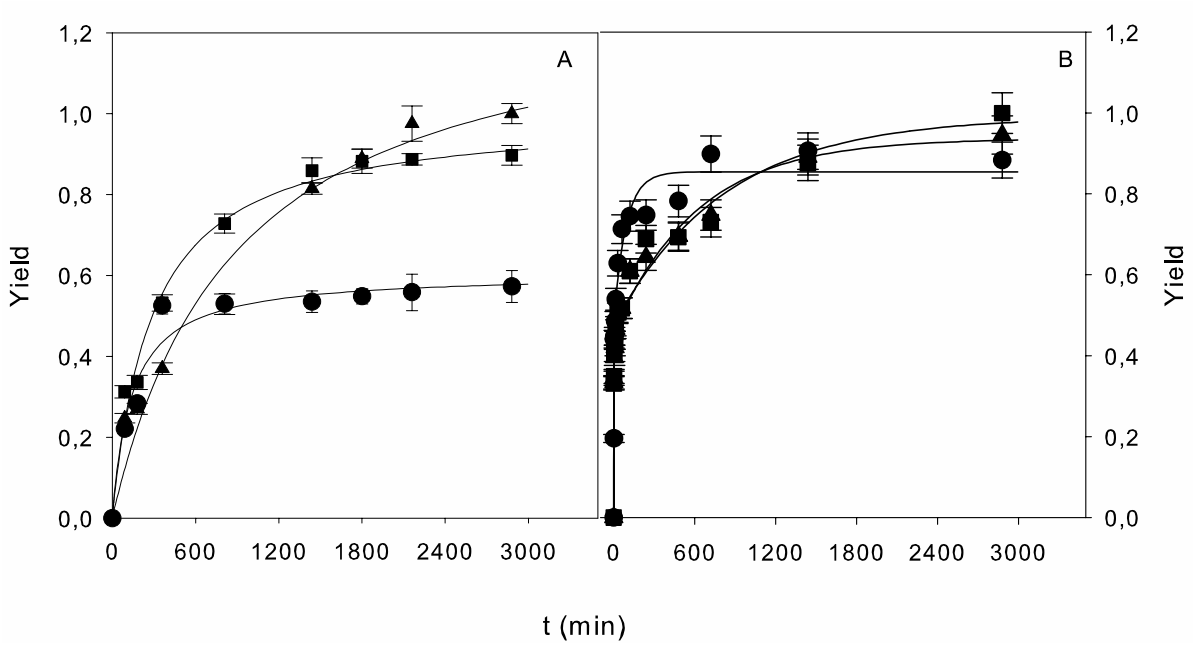


Figure 6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0
600
1200
1800
2400
3000
t (min)

Table
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Tables

Table 1

Coded values	Natural values		
	T (ºC)	t (min)	R (L:L or L:S)
-1.68 (-α)	40.0	30	1.0
-1	52.2	85	1.4
0	70.0	165	2.0
+1	87.8	245	2.6
+1.68 (+α)	100.0	300	3.0

Codification: $V_c = (V_n - V_0)/\Delta V_n$
Decodification: $V_n = V_0 + (\Delta V_n \times V_c)$
Vn = natural value in the centre of the domain
ΔV_n = increment of V_n for unit of V_c

Table 2

T	*t*	*R*	*Y*	*Ye*	Coefficients	t-Student	Model
-1	-1	-1	8.16	8.09	8.23	35.92	**8.23**
1	-1	-1	6.96	8.38	-1.53	10.08	**-1.53T**
-1	1	-1	9.61	9.22	0.06	0.41	t(NS)
1	1	-1	1.69	2.79	0.69	4.53	**0.69R**
-1	-1	1	7.81	8.28	-1.15	5.81	**-1.15Tt**
1	-1	1	5.97	6.48	0.37	1.84	TR(NS)
-1	1	1	11.61	11.77	1.11	5.61	**1.11tR**
1	1	1	7.26	7.45	0.53	2.64	**0.53TtR**
-1.682	0	0	8.95	9.61	-0.43	2.88	**$-0.43T^2$**
1.682	0	0	5.59	4.45	-0.07	0.44	t^2(NS)
0	-1.682	0	8.41	8.23	-0.19	1.26	R^2(NS)
0	1.682	0	8.17	8.23			
0	0	-1.682	7.07	7.07			
0	0	1.682	8.90	9.39	Average value	7.77	
0	0	0	7.22	8.23	Expected average value	8.22	
0	0	0	8.08	8.23	Var (E_e)	0.316	
0	0	0	8.31	8.23	t (α<0.05; ν=6)	2.447	
0	0	0	8.79	8.23			
0	0	0	8.22	8.23			
0	0	0	8.69	8.23			

	SS	ν	ν	QM	Mean square ratios	
Model (M)	64.62	-	6	10.770	QM_M/QME= 18.6	$F_{13}^{6}(\alpha = 0.05) = 2.915$
Error (E)	7.53	-	13	0.579	$QM_{(M+LF)}/QM_M$= 0.504	$F_{6}^{13}(\alpha = 0.05) = 3.976$
Exp. Error (E_e)	1.578	6	-	0.263	QM_E/QM_{Ee}= 2.201	$F_{6}^{13}(\alpha = 0.05) = 3.976$
Lack of Fit (LF)	5.95	7	-	0.850	QM_{LF}/QM_{Ee}= 3.231	$F_{6}^{7}(\alpha = 0.05) = 4.207$
Total	72.15		19		R^2= 0.896 adjusted R^2= 0.847	

Y: observed response; Y_e: expected response; NS: non signifcant coefficient; SS: sum of squares; ν: degrees of freedom; QM: quadratic means of model (M), total error (E), experimental error (E_e) and lack of fit (LF). Independent variables according to Table 1.

Table 3

Model	Parameters	R_f			HR_f		
		1:1	2:1	3:1	1:1	2:1	3:1
[3]	Y_e	0.72±0.04	0.87±0.02	0.97±0.04	0.94±0.06	0.97±0.04	0.94±0.05
	k_La	0.0057±0.0015	0.0103±0.0013	0.0072±0.0013	0.0083±0.0027	0.0118±0.0031	0.0080±0.0022
	R^2	0.985	0.996	0.990	0.970	0.984	0.978
[4]	Y_e	0.79±0.03	0.91±0.04	1.00±0.04	1.00±0.03	1.00±0.02	1.00±0.03
	k_A	0.0116±0.0029	0.0212±0.0013	0.0131±0.0035	0.0132±0.0031	0.0235±0.038	0.0130±0.0032
	R^2	0.994	0.988	0.997	0.995	0.999	0.994

Table 4

Model	Parameters	Temperature (ºC)			
		30	40	50	60
[4]	Y_e	0.87±0.06	0.59±0.08	0.43±0.08	0.29±0.01
	k_A	0.38±0.17	0.28±0.26	0.48±0.43	1.98±0.58
	R^2	0.963	0.800	0.681	0.978
[5]	Y_m	0.89±0.13	0.77±0.04	0.61±0.04	0.29±0.01
	Y_e	0.44±0.10	0.37±0.02	0.26±0.02	0.18±0.049
	k_{L1}	1.23±0.17	0.46±0.12	0.91±0.29	0.96±0.58
	k_{L2}	0.0373±0.0051	0.0015±0.0005	0.0013±0.0004	0.0571±0.0420
	R^2	0.975	0.993	0.993	0.988

Table 5

Sample	Parameters	Treatment		
		Control	BHA	ETQ
R_f	Y_e	0.61±0.06	1.00±0.07	1.32±0.30
	k_A	0.0117±0.0072	0.0035±0.0013	0.0009±0,0007
	R^2	0.963	0.989	0.976
LR_f	Y_{max}	0.85±0.08	0.99±0.12	0.94±0.08
	Y_e	0.37±0.13	0.52±0.12	0.48±0.08
	k_{L1}	0.0110±0.0083	0.0012±0.0007	0.0016±0.0008
	K_{L2}	0.59±0.37	0.72±0.34	0.78±0.34
	R^2	0.958	0.974	0.981

Abstract

A protein and astaxanthin- concentrated fraction (R_f) can be recovered from shrimp cooking wastewater by ultrafiltration at 300 kDa, indicating astaxanthin is somehow associated to membrane- retained proteins. Concentrated astaxanthin from shrimp wastewater can be extracted using sunflower oil under milder conditions (T<40ºC) than directly from shrimp exoskeleton. Modeling astaxanthin extraction kinetics at 30ºC revealed the process is consequence of both mass transfer and hydrogen bonding between astaxanthin and oil. The best yields of astaxanthin extraction were obtained using an oil:waste ratio of 3:1 which was not further improved after hydrolysis with alcalase at 45ºC for 30 min (HR_f). The lyophilized concentrate (LR_f) showed two-phase extraction profiles with a much faster pigment recovery observed at 30ºC compared to the liquid form. Astaxanthin from this shrimp by-product has low thermal stability in oil at high temperatures (60 and 70ºC), suggesting the carotenoid is mainly free as a result of the cooking process and not bounded to proteins or lipids as it occurs in its natural form.